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**(54) Title:** PROCESS FOR THE PRODUCTION OF POLY(HYDROXY FATTY ACIDS) AS WELL AS RECOMBINANT BACTERIAL STRAINS FOR CARRYING OUT THE PROCESS

**(57) Summary**

The present invention relates to a process for the production of poly(hydroxy fatty acids) as well as recombinant bacterial strains for carrying out the process. In addition, new poly(hydroxy fatty acids) and new substrates for the production of conventional and new poly(hydroxy fatty acids) are described. Moreover, the invention also relates to a DNA fragment, which codes for a PhaE and a PhaC component of the poly(hydroxy fatty acid) synthase from Thiocapsa pfennigii, as well as the corresponding poly(hydroxy fatty acid) synthase.[1]

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[1] [Translator's note: please see the title and summary in European English; these are not quite the same as the

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## Specification

# Process for the production of poly(hydroxy fatty acids) as well as recombinant bacterial strains for carrying out the process

The present invention relates to a process for the production of poly(hydroxy acids) in accordance with Claim 1, a recombinant bacterial strain in accordance with Claim 19, a poly(hydroxy fatty acid) in accordance with Claim 23 and a DNA fragment in accordance with Claim 33.

In this age of increasing environmental awareness, there are increasing attempts in industry and science to produce biodegradable polymers. In this regard, these new types of environmentally compatible polymers should essentially have the same properties as those polymers which, for decades, have been prepared via organic chemical synthesis.

In particular in this connection, the ability to process the new types of biodegradable polymers ought to be provided [in a similar manner to] the processing of conventional plastics using the same methods such as, for example, extrusion, injection molding, injection compression, foaming, etc.

A big disadvantage of organically synthesized plastics is, however, that many of these plastics have enormous biological half-lives or, as the case may be, they cannot be disposed of in garbage dumps or in garbage incineration plants in a non-harmful manner but, rather, aggressive gases are frequently produced such as, for example, in the case of poly(vinyl chloride) which liberates hydrogen chloride gas during incineration.

A first step in the direction of success with environmentally compatible materials was achieved by means of synthetic substances, e.g. the paraffin-like polymers polyethylene and polypropylene since these essentially release only $CO_2$ and water on incineration.

In addition, many attempts have also been made by means of so-called replaceable raw materials such as, e.g. plants that contain a lot of polysaccharide such as potatoes, corn, wheat, beans, peas or similar materials, to obtain the naturally occurring polysaccharides in these plants and to prepare polymers from them which are usable in plastics technology and which are biodegradable.

However, in the case of such polymer materials comprising replaceable raw materials, one is essentially relying on the natural quality of the polymers that occur in these higher plants and only the relatively complex processes of classical cultivation and modern gene technology offer themselves for modification at the genetic level.

An essential further step in the direction of naturally occurring polymers, which are very similar to synthetic thermoplastics, was brought about by the discovery of poly(3-hydroxybutyric acid) by Lemoigne in 1926 [Lemoigne, M. (1926) Products of the dehydration and polymerization of b-oxybutyric acid, Bull. Soc. Chim. Biol. (Paris) 8: 770-782]. The discovery by Lemoigne can be considered to have paved the way for the further development of modern poly(hydroxy fatty acids) which are also designated polyhydroxyalkanoates and represent chemically linear esters of hydroxy fatty acids and hence, ultimately, polyesters.

In the eighties and, especially, in the last five years, further hydroxy fatty acids have been described as components of the poly(hydroxy fatty acids) (PHF) that occur in nature. In this connection, the hydroxyl group of these PHF is usually located in the 3' position. The aliphatic side chains are either saturated or singly or doubly unsaturated. They are thus non-branched or branched and they can be substituted by functional groups such as, for example, halogen atoms, preferably bromine, iodine and chlorine, or cyano groups, ester groups, carboxyl groups or even cyclic aliphatic groups

and even aromatic groups. In some hydroxy fatty acids, the hydroxyl group is also located in the 4' or 5' position.

Poly(hydroxy fatty acids) have been detected previously in gram positive and gram negative groups of bacteria, aerobic and anaerobic groups of bacteria, heterotrophic and autotrophic groups of bacteria, eubacteria and archaebacteria and in anoxygenic and oxygenic photosynthetic groups of bacteria and therefore in virtually all important groups of bacteria. Thus the capability of synthesizing such polyesters apparently does not represent any specially demanding or rare biochemical metabolism. Biosynthesis of the PHF usually sets in when a usable source of carbon is present in excess with the simultaneous deficiency of another nutrient component. In this way, a nitrogen deficiency, a phosphorus deficiency, a sulfur deficiency, an iron deficiency, a potassium deficiency, a magnesium deficiency or an oxygen deficiency can trigger PHF synthesis in bacteria [Anderson, A.J. and Dawes, E.A. (1990) Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates, Microbiol. Rev. 54: 450-472; Steinbüchel, A. (1991) Polyhydroxyalkanoic acids: In: D. Byrom (editor) Biomaterials, Macmillan Press, New York, pages 123-213]. In most bacteria, PHF are deposited in the form of inclusions or grana in cytoplasm, whereby the dry mass of the cell can amount to up to a proportion of 95% by weight.

In eukaryotes, only poly(3-hydroxybutyric acid) has previously been demonstrated as the single PHF. This polyester arises in yeasts such as, for example, Saccharomyces cerevisae, various plants, e.g. cauliflower, various organs from animals, e.g. the liver and also in humans, e.g. in blood plasma [Reusch, R.N. 1992, Biological complexes of polyhydroxybutyrate, FEMS Microbiol. Rev. 103: 119-130]. However, in contradistinction to prokaryotes, the proportion of poly(3-hydroxybutyric acid) in eukaryotes is maximally 0.1% by weight. Inclusions in the form of grana, in the manner in which they occur in prokaryotes, are not known in eukaryotes. As a rule, the eukaryotic PHF are not usually present in free form, either but the polyester is present either linked to other proteins or in the form of a complex which spans the cytoplasm membrane together with calcium ions and polyphosphate molecules.

Thus, only the production of PHF in bacteria is of interest for industrial biotechnological purposes.

The biosynthesis of PHF in bacteria can be subdivided into three phases.

In phase I, the carbon source, which is offered to the bacteria in the medium, is first taken up in the bacterial cells. Either special uptake transportation systems have to exist for the corresponding carbon source or the cells are cultivated under conditions which produce a certain artificial permeability of the cytoplasm membrane with respect to the carbon source. Some non-ionic carbon sources, for example fatty acids in their non-dissociated form, can also get into the cells via passive diffusion.

In phase II, the absorbed carbon source is transformed into a suitable substrate for the particular enzyme which is capable of producing PHF. This enzyme is generally designated poly(hydroxy fatty acid) synthase. Here, numerous more or less complex reaction sequences are conceivable, which can include both anabolic enzymes and catabolic enzymes in the reaction pathway, and these have been demonstrated already, too.

Phase III comprises the linking together of monomeric precursors to give the polyester. This reaction is catalyzed by the enzyme PHF synthase which represents the key enzyme for the biosynthesis of PHF. These enzymes are linked to the PHF grana and they are located there on the surface. Engendered by the very low specificity of most of the PHF synthases that have previously been examined in this regard and which arise in differing species, the biosynthesis of a plurality of different PHF is possible. Previously, only the co-enzyme A-thioesters of hydroxy fatty acids have been detected in the form of monomeric, bio-synthetically active precursors. As has been shown above, PHF synthase is the key enzyme for PHF synthesis.

After the structure gene of the PHF synthase from Alcaligenes eutrophus had been cloned and synthesized in three different laboratories independently of one another, the structure genes for the

Dennis, D.E. (1988) Cloning and expressing Escherichia coli of the Alcaligenes eutrophus H16 poly-b-hydroxybutyrate bio-synthetic pathway, J. Bacteriol. 170: 4431-4436; Schubert, P., Steinbüchel, A. and Schlegel, H.G. (1988) Cloning of the Alcaligenes eutrophus gene for synthesis of poly-b-hydroxybutyric acid and synthesis of PHB in Escherichia coli, J. Bacteriol. 170: 5837-5847; Peoples, O.P. and Sinslkey, A.J. (1989) Poly-b-hydroxybutyrate biosynthesis and Alcaligenes eutrophus H16. Identification and characterization of the PHB polymerase gene (*phbC*), J. Biol. Chem. 264: 15298-15303].

At that time, the nucleotide sequences of at least 12 poly(hydroxy fatty acid) synthase genes (PHF synthase genes) were determined. Because of the primary structures of the enzymes, that were derived from this, and because of physiological data, three different types of PHF synthases can now be distinguished. Type I is represented by the PHF synthase from the Alcaligenes eutrophus bacterium which has been examined most thoroughly of all in regard to PHF metabolism and which has a molecular weight of 63,940 and catalyzes the synthesis of PHF from hydroxy fatty acids with a short chain length. In addition to 3-hydroxy-propionic acid, 3-hydroxybutyric acid and 3-hydroxyvaleric acid, 4-hydroxybutyric acid, 4-hydroxyvaleric acid and 5-hydroxyvaleric acid are also incorporated into a copolyester comprising different hydroxy fatty acid subunits.

Type II is represented by the PHF synthase from Pseudomonas oleovarans. This enzyme has a similar size to that of the type I PHF synthases (molecular mass 62,400); however, it differs considerably relative to the substrate specificity of the type I PHF synthases. It is capable of incorporating only 3-hydroxy fatty acids of medium chain length into PHF. 4-hydroxy fatty acids and 5-hydroxy fatty acids and 3-hydroxybutyric acid, by contrast, are not incorporated into the bio-synthetic polyesters. However, the specificity of the enzyme is still so broad that approximately 50 different 3-hydroxy fatty acids can be processed as substrates.

Type III is represented by the PHF synthase from Chromatium vinosum. This enzyme resembles the type I PHF synthases from the point of view of substrate specificity. However, it has a

distinctly lower molecular mass (approximately 39,730) and needs a second protein in order to be catalytically active.

To the extent that PHF have previously been isolated from bacteria, these do have extremely interesting properties: they are thermoplastically deformable, water-insoluble, biodegradable, non-toxic and optically active provided that they are not homopolyesters of w-fatty acids. It has also been shown in the case of poly(3-hydroxybutyric acid) that it is bio-compatible and that it has piezoelectric properties.

It has been shown for poly(3-hydroxybutyric acid) [poly(3HB)] and for the copolyester poly(3-hydroxybutyric acid-co-3-hydroxy-valeric acid) [poly(3HB-co-3HV)] that these polymers can be processed with conventional injection molding processes, extrusion blowing processes and injection blowing processes as well as by fiber spinning techniques.

Only two poly(hydroxy fatty acids), namely the homopolyester poly(3HB) and the copolyester poly(3HB-co-3HV), have advanced thus far to large scale production maturity. The copolymer is marketed under the trade name "Biopol".

The production of these biopolymers is disclosed in EP-A 69 497. Production of the polymer is carried out in the form of a two-stage fed-batch process in a 35 $m^3$ air-lift reactor and in tubular kettle reactors with working volumes of up to 200 $m^3$ with a double mutant of Alcaligenes eutrophus as the production organism and with glucose and propionic acid as the carbon sources together with phosphate limitation [Byrom, D. (1990) Industrial production of copolymer from Alcaligenes eutrophus. In: Dawes, E.A. (editor) Novel biodegradable microbial polymers, pages 113-117, Kluwer Academic Publishers, Doordrecht]. The first stage serves for the growth of bacterial cells to high densities and lasts approximately 48 hours, whereby only glucose is offered as the substrate. In the second stage, the cells are grown with phosphate limitation and with glucose and propionic acid as the precursors for the 3-hydroxyvaleric acid component; cell densities

achieved after a further 40 to 50 hours of cultivation. The cells are then treated with an enzyme cocktail, which essentially comprises lysozyme, proteases and other hydrolytic enzymes, as a result of which the PHF grana are released. The grana sediment on the bottom of the reactor and are collected from there, washed, dried, melted, extruded and granulated.

This PHF is currently produced in a production quantity of approximately 300 metric tons on an annual basis. Although these microbially produced biopolymers, poly(3HB) and poly(3HB-co-3HV), have good properties and can be processed with the methods that are usual in plastics technology, their production is, on the one hand, still very expensive and, on the other hand, it [translator: i.e. the copolymer] contains only two monomeric sub-units so that the total properties of the polymer, that is produced, can be controlled only via these two quantities and thus precise control in regard to flexibility, processability in plastics technology plants, resistance to certain solvents, etc. cannot be carried out in fine controlling steps.

Although 3-hydroxyvaleric acid confers good flexibility or, as the case may be, processability on PHF, it has been found that, for example, the component 4-hydroxyvaleric acid, which can additionally be present in the PHF which are synthesized by bacteria, confers on the biopolymer, that is produced, a distinctly higher degree of flexibility than is the case with 3-hydroxyvaleric acid alone.

In the prior art, 4-hydroxyvaleric acid (4HV) has been demonstrated as a new component in bacterial PHF. Various bacteria were capable of synthesizing polyesters with this new component. These are usually copolyesters which also contain 3-hydroxybutyric acid and 3-hydroxyvaleric acid as components in addition to 4HV. However, these terpolymers could previously be produced only starting out from expensive and toxic special chemicals which were offered to the bacteria as precursor substrates or, as the case may be, as a carbon source for PHF biosynthesis.

In particular, Valentin, H.E., Schönebaum, A. and Steinbüchel, A. (1992) Appl. Microbiol.

polyhydroxyalcanoic acids from bacteria" describe the manufacture of a terpolyester, which consists of 3-hydroxybutyric acid, 3-hydroxyvaleric acid and 4-hydroxyvaleric acid as subunits, whereby, for example, 4-hydroxyvaleric acid or 4-valerolactone is offered to an Alcaligenes strain as the sole carbon source in a batch process, a fed-batch process or a two-step batch process.

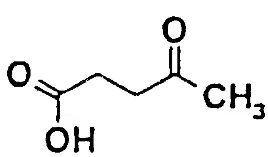
However, in order to move the microorganisms, which are used in this prior art, to incorporate 4-hydroxyvaleric acid, the prior art requires the very expensive and toxic 4-hydroxyvaleric acid itself or its lactones.

Starting out from this prior art, the problem for the present invention was therefore to make PHF available with improved properties and with cheaper and non-toxic starting substances.

From a process technical standpoint, the above problem is solved by the characterizing features of Patent Claim 1.

In regard to the recombinant bacterial strain, the problem is solved in accordance with the characterizing features of Patent Claim 19. In regard to the poly(hydroxy fatty acid), the above problem is solved by the characterizing features of Patent Claim 23. In addition, the DNA fragment in accordance with Patent Claim 33 also solves the above problem.

In accordance with the process of the invention and in accordance with Claim 1, it has been possible for the first time to produce 4HV-containing polyesters starting out from levulinic acid. The chemical structure of levulinic acid is reproduced in the following formula:



Levulinic acid (4-oxopentanoic acid)

Seen chemically, levulinic acid is 4-oxopentanoic acid which is readily soluble in water, alcohol and ether.

This is a relatively inexpensive substance, since it can be prepared from hexoses of plant origin -- i.e. replaceable raw materials -- e.g. by boiling with hydrochloric acid. In addition, it is also generated in a large quantity in the form of a waste product during the processing of wood and it can thus be processed further on a large industrial scale. The PHF-free mutants GPp104 of Pseudomonas putida (Huismann, G.W., Wonink, E., Meima, R., Kazemier, W., Terpstra, P. and Witholt, B. (1991) J. Biol. Chem. 266: 2191-2198) and PHB$^-$4 of Alcaligenes eutrophus H16 (Schlegel, H.G., Lafferty, R. and Krauss, I. (1970) Arch. Microbiol. 71: 283-294) are used as production organisms in accordance with the present invention into which the plasmid pHP1014::E156, which, inter alia, contains and expresses the structure group of the PHF synthase from Thiocapsa pfennigii, had previously been conjugatively transferred (Liebergesell, M., Mayer, F. and Steinbüchel, A. (1993) Appl. Microbiol. Biotechnol. 40: 292-300; Valentin, H.E., Lee, E.Y., Choi, C.Y. and Steinbüchel, A. (1994) Appl. Microbiol. Biotechnol. 40: 710-716). In the process in accordance with the invention, use is also made of the new organisms Pseudomonas putida GPp104 (pHP1014::B28+), which was officially filed by BUCK-Werke GmbH & Co., Geislinger Str. 21, 73337 Bad Überkingen, Germany, at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig under No. 9417 (Pseudomonas putida SK 6691 - DSM 9417) on 09.05.1994 in accordance with the Budapest treaty, and Alcaligenes eutrophus PHB$^-$4 (pHP1014::B28+) which was officially filed at the Deutsche Sammlung von Mikroorganismen [German Collection of Microorganisms], Braunschweig, under No. 9418 (Alcaligenes eutrophus SK 6891 - DSM 9418) on 09.05.1994 in accordance with the Budapest treaty.

These strains have the special property that they contain essentially exactly those DNA fragments which contain and express the genes *phaE* and *phaC*; this is because the gene products *phaE* and *phaC* are together capable of revealing PHF synthase activity.

Of course, it is well known to someone who is skilled in the art that nucleotide sequences, which carry *phaE* and *phaC* genes additionally have conventional control regions, e.g. promoters, S/D sequences or similar entities.

Using the process in accordance with the invention, it is possible to produce new 4HV-containing copolyesters which also have thermoplastic properties and which behave distinctly more flexibly than the copolyesters of the prior art.

Because of the inexpensive starting material levulinic acid which is used, the polymers could also be produced distinctly more cheaply than had previously been possible with the biotechnological processes of the prior art.

Instead of levulinic acid, use can also be made, of course, of a salt of levulinic acid or, as the case may be, a lactone of levulinic acid or, as the case may be, other derivatives, e.g. halogen derivatives, as the substrate for the production organisms of the present invention.

In addition, the inventors of the present invention have found that the recombinant bacterial strains which contain and express at least a fragment of the gene of the PHF synthase from Thiocapsa pfennigii are also capable of incorporating 5-hydroxy-hexanoic acid, its salts, esters and lactones into a copolyester which has been bio-synthesized by these bacteria. The preparation of 5-hydroxyhexanoic acid took place starting from 4-acetylacetic acid, which was reduced quantitatively using $NaBH_4$. The detection of 5-hydroxyhexanoic acid (5HHx) as a component took place gas chromatographically after methanolysis both in lyophilized cells and in the isolated and purified polyester. The isolated and purified polyester was subjected to $^{13}C$-NMR analysis and $^{1}H$-NMR analysis and the incorporation of 5 HHx was confirmed as a result of this.

By way of example, the analysis of the polyester, that was accumulated by the bacterial cells, resulted in a polyester content of more than 40% by weight of the dry mass of the cells, whereby a

approximately 71 mol% of 3-hydroxybutyric acid, approximately 4 mol% of 3-hydroxyhexanoic acid, approximately 23 mol% of 5-hydroxyhexanoic acid and approximately 2 mol% of 3-hydroxy-octanoic acid.

Numerous wild type strains were also examined in regard to their capacity for being able to bio-synthesize PHF-polyesters with 5 HHx as a subunit, starting from 5-hydroxyhexanoic acid as the carbon source. However, none of the tested strains were capable in this regard.

If 4-hydroxyheptanoic acid (4HHp) is offered as the source of carbon to the recombinant bacterial strains, that were used for the present invention, then one also finds these new components in the form of a subunit in the copolyester that is synthesized by the bacteria.

The preparation of 4-hydroxyheptanoic acid took place via the hydrolysis of g-heptalactone with NaOH.

By way of example, the analysis of the polymer that was accumulated by the recombinant cells resulted in a polyester content of approximately 40% by weight of the dry mass of the cells. A typical polymer contained approximately 43 mol% of 3-hydroxybutyric acid, approximately 16 mol% of 3-hydroxyvaleric acid, approximately 27 mol% of 3-hydroxyhexanoic acid, approximately 5 mol% of 3-hydroxyheptanoic acid, approximately 6 mol% of 4-hydroxyheptanoic acid and approximately 6 mol% of 3-hydroxyoctanoic acid.

Numerous wild type strains were examined in regard to their capacity for being able to bio-synthesize copolyesters with 4HHp as a component starting from 4-hydroxyheptanoic acid as the carbon source; however, none of the wild type strains, which were tested, was capable in this regard.

Here also, the detection of 4HHp took place gas chromatographically after methanolysis both in lyophilized cells and also using isolated and purified polyesters.

The inventors have also found that a recombinant strain, which contains the gene of the PHF synthase from Thiocapsa pfennigii e.g. a PHF-free mutant GPp104 of Pseudomonas putida, can synthesize a new copolyester that contains the 4-hydroxyoctanoic acid component (4HO).

The detection of 4HO took place gas chromatographically after methanolysis both in lyophilized cells and also using isolated and purified polyesters.

The preparation of 4-hydroxyoctanoic acid takes place via the hydrolysis of g-lactone with NaOH.

Typically, the bacterial cells accumulated the synthesized copolyester up to a concentration of approximately 20% by weight of the dry mass of the cells. The polymer contained, for example:

approximately 75 mol% of 3-hydroxybutyric acid, approximately 22 mol% of 3-hydroxyhexanoic acid, approximately 1.5 mol% of 4-hydroxyoctanoic acid and approximately 3 mol% of 3-hydroxy-octanoic acid.

In this regard also, numerous wild types of strains were also examined in regard to their capacity for bio-synthesizing copolyesters with 4HO as a subunit starting from 4-hydroxy-octanoic acid as the carbon source; however, none of the tested wild strains were capable of this.

The new copolyesters, that are prepared by means of the process in accordance with the invention also exhibit thermoplastic properties and they can be processed in a problem-free manner using the techniques which are conventional in plastics technology.

These were water-insoluble thermoplastic copolymers that have a high degree of bio-compatibility which makes these materials appear to be usable for application in medical technology -- e.g. as implants -- or as suture material or similar articles.

Basically, the time of cultivation of the microorganisms, which are used for the purposes of the present invention, is governed by the culture conditions which are governed primarily by the temperature, the oxygen content of the medium (aerobic conditions) and by the medium itself, the quantity of the carbon source, the mineral salts, the trace elements and/or the pH value. The quantity of the substrates used in each case is governed by the microorganism in question. However, one can start from concentrations in the range from approximately 0.1% (weight/volume) up to 10% (weight/volume) corresponding to 100 g/l or, especially, 0.2% (weight/volume) up to 5% (weight/volume).

The harvesting of the cells can generally take place during the log phase [translator: lag phase?] up to the stationary phase; it should preferably take place in the stationary phase. The bacterial cells can be obtained from the medium in their entirety either after single culturing (batch process or fed-batch process) or they can be obtained continuously via continuous culturing, e.g. by means of conventional centrifugation or filtration processes.

After optionally washing, for example with a buffer, preferably a phosphate buffer or, especially preferably, a sodium phosphate buffer in the neutral region of approximately pH 7.0, the harvested cells can be frozen, lyophilized or treated by means of spray drying.

Obtaining the polyesters in accordance with the invention can take place in accordance with known methods; dissolution or extraction is preferably carried out with organic solvents, especially by means of halogenated or, preferably, chlorinated hydrocarbons or, especially preferably, by means of chloroform or methylene chloride.

The copolyesters, that are obtained in accordance with the invention, are easy to process in the form of thermoplastics and are usable in many ways. For example, in surgery, for articles for closing wounds, e.g. as suture material or clamps or similar articles, as an attachment element for bones, e.g. fixation pins, plates, screws, dowels, as a separating material, filling material or covering material, e.g. in the form of fabric, fleece or wadding. Likewise, the polyesters in accordance with the invention can be used in pharmaceutical galenic preparations, e.g. in the form of ancillary substances, carrier materials, release systems for medicinal agents or for the encapsulation and/or micro-encapsulation of substances and active materials.

In addition, the preparation of biodegradable packaging materials such as foils, bottles, ampoules, cans, pouches, boxes, cases or similar items is also possible by means of the present invention.

The recombinant bacteria, which are to be pre-cultivated in a complex medium in accordance with Claim 2, have the advantage that, as a result of this, intense multiplication of the biomass is achieved initially in order then to stimulate the bacteria biochemically to bio-synthesize the desired PHF.

An additional carbon source in accordance with Claim 3 to promote growth which is to be added to the nutrient medium for the culturing of the bacteria has the advantage that, as a result of this, various subunits can be incorporated on the one hand and, on the other hand, the bacteria grow considerably faster in part and, at the same time, they bio-synthesize a larger quantity of the desired PHF.

The procedures of Claim 4 have the advantage that the process of the present invention can be carried out using processes that are conventional in large scale industrial biotechnology.

The procedures of Claim 5 have the advantage that an economical ratio of the polyester yield to the dry mass of the bacterial cells can be obtained so that, seen economically, the yield obtained is

Profitability calculations have shown that the lower limit for profitability can lie in the order of approximately 30% by weight of polyester based on the dry mass of the bacterial cells.

However, using the present invention, it is possible to achieve values which are distinctly in excess of 40% PHF based on the dry mass of the bacterial cells.

In this way, the yield can, of course, be increased considerably further by suitable alteration of the biochemical and/or biophysical parameters in order to control the biotechnological process, e.g. pH adjustment, pressure and/or temperature adjustment, step-wise addition of the substrates and/or the substrate mixtures, cell densities, nutrient medium compositions, etc.

The procedures of Claim 6 have the advantage that, in the case of copolyesters with at least two subunits, the chemical, biochemical and physical properties of the polyester can be adjusted by varying the different subunits in fine steps if one offers the appropriate substrates to the bacteria.

Allowing the recombinant bacteria to grow to cell densities of up to 100 g of cellular dry mass per liter of bacterial nutrient medium in accordance with Claim 7, has the advantage that, together with appropriate dimensions for large scale industrial plants, relatively small volumes contain a considerable biomass and thus increase the productivity significantly relative to lower cell densities.

To offer the substrate carbon source in an excess quantity in accordance with Claims 8 and 9 has the advantage that the substrate is then taken up preferentially by the bacteria because of the concentration gradient and is then used for the production of the polyester.

The procedures of Claim 10 have the advantage that, as a result of the step-wise increase in the concentration of the substrate carbon source, the total process can be controlled better in the direction of higher yields.

The procedures of Claims 11, 12 and 13 give advantageous process conditions for the biotechnological preparation of PHF in accordance with the present invention.

The procedures of Claims 14 and 15 have the advantages that all the usual methods in biotechnology for breaking open bacteria can be used in order to obtain the bio-industrially produced PHF.

Since these are generally heavier than the nutrient medium and the cell debris that surround them, the PHF can easily be separated and obtained by accelerated sedimentation, for example by centrifugation.

The procedures of Claim 16 have the advantage that, as a result of the introduction of a PHF product, that has been dissolved by means of an organic solvent, in water or a lower alcohol, preferably ethanol, the PHF are precipitated and a purification step is consequently achieved which can be compared with a recrystallization process in organic chemistry for the purification of the desired product.

To use an enzyme cocktail in accordance with Claims 17 and 18 has the advantage that here, specifically, the bacterial cell wall and the membrane are destroyed enzymatically so that the grana, which contain the PHF, are precipitated from the cytoplasm and sediment on the bottom of the reactor. Since, especially preferably, proteolytic and lytic enzymes, e.g. lysozymes or even lipases, are used as a rule in this regard, the entire biotechnological preparation essentially comprises macromolecules -- namely the desired polyester which is being synthesized -- as well as a plurality of smaller molecules which arise as a result of the enzymatic cleavage of the nucleic acids, proteins glycoproteins, polysaccharides and lipids, that are contained in the cells and the cell walls and cell membrane, and which can thus be separated from one another with ease without the isolated PHF containing significant impurities from other bacterial compounds.

The use of detergents is especially advantageous in this connection since proteins and nucleic acids are also solubilized, in particular, as a result of this and they are then degraded in the form of quasi colloid particles which are suspended in the aqueous solutions of the other enzymes.

In this connection, note must of course be taken of the fact that detergents are applied in which the enzymes that are used are still very active or are very active for the first time. Such a mild detergent is, for example, octyl glucoside. On the other hand, it is known, for example in regard to the V8 protease from Staphylococcus aureus, that it still reveals intense proteolytic activity in 1 to 2% sodium dodecyl sulfate.

Claims 19 to 21 relate to new recombinant bacterial strains in accordance with the invention which contain and express the genes *phaC* and *phaE* from Thiocapsa pfennigii which are relevant for PHF synthesis.

The *Bam*HI fragment B28, that is contained in the newly constructed bacterial strains in accordance with the invention, was obtained following *Bam*HI digestion of the *Eco*RI fragment E156 and essentially comprises the two genes *phaC* and *phaE*.

The bacterial strains in accordance with the invention and in accordance with Claim 19 generate especially high yields of PHF.

Claim 23 relates to a PHF in the form in which it is obtainable in accordance with a process according to one of the Claims 1 through 18; in particular, the following PHF or, as the case may be, polyesters or, as the case may be, copolyesters could be obtained in accordance with Claim 24 using the process in accordance with the invention:

(A) 3-hydroxybutyric acid, 3-hydroxyvaleric acid and 4-hydroxy-valeric acid;

(B) 3-hydroxybutyric acid, 3-hydroxyvaleric acid, 4-hydroxy-valeric acid, 3-hydroxyhexanoic acid and 3-hydroxyoctanoic acid;

(C) 3-hydroxybutyric acid, 3-hydroxyhexanoic acid, 5-hydroxy-hexanoic acid and 3-hydroxyoctanoic acid;

(D) 3-hydroxybutyric acid, 3-hydroxyvaleric acid, 3-hydroxy-hexanoic acid, 3-hydroxyheptanoic acid, 4-hydroxyheptanoic acid and 3-hydroxyoctanoic acid;

(E) 3-hydroxybutyric acid, 3-hydroxyhexanoic acid, 3-hydroxy-octanoic acid and 4-hydroxyoctanoic acid;

(F) 3-hydroxybutyric acid, 3-hydroxyhexanoic acid and 5-hydroxy-hexanoic acid;

(G) 3-hydroxybutyric acid, 3-hydroxyvaleric acid, 3-hydroxy-heptanoic acid and 4-hydroxyheptanoic acid;

(H) 3-hydroxybutyric acid, 3-hydroxyvaleric acid, 3-hydroxy-hexanoic acid, 3-hydroxyoctanoic acid and 4-hydroxyoctanoic acid;

(I) 3-hydroxybutyric acid, 3-hydroxyhexanoic acid and 4-hydroxy-hexanoic acid;

(J) 3-hydroxybutyric acid and 5-hydroxyhexanoic acid.

Claims 25 through 32 indicate preferred quantitative compositions of the PHF in the form in which they are obtained by the process in accordance with the invention. All the PHF that are obtained are capable of being processed as a thermoplastic and exhibit high flexibility.

Claims 33 and 34 relate to a DNA fragment which carries the genes *phaE* and *phaE* from Thiocapsa pfennigii. The *phaC* gene codes for the phaC protein and the *phaE* gene codes for the phaE protein.

The two proteins together exhibit PHF synthase activity.

Further advantages and characteristic features of the present invention arise on the basis of the description of the examples and on the basis of the drawing. The following aspects are shown:

Figure 1 shows the DNA sequence of a DNA fragment in accordance with the invention from Thiocapsa pfennigii and the amino acid sequence of the phaC and phaE proteins.

The 2.8 kb DNA fragment, that is shown in Figure 1, is obtained via the *Bam*HI digestion of a 15.6 kb *Eco*RI fragment from Thiocapsa pfennigii. In addition, Figure 1 shows the assignment of the amino acid sequences of the phaC and phaE proteins (using the IUPAC one letter code) to their corresponding genes *phaC* (DNA sequence section 1322 to 2392) and *phaE* (DNA sequence section 180 to 1280).

## Example 1

8 l of mineral salt medium (Schlegel, H.G., Kaltwasser, H. and Gottschalk, G., 1961, Arch. Microbiol. 38: 209-222) with the composition

| | |
|---|---|
| $Na_2HPO_4 \times 12H_2O$ | 9.0 g |
| $KH_2PO_4$ | 1.5 g |
| $NH_4Cl$ | 0.5 g |
| $MgSO_4 \times 7 H_2O$ | 0.2 g |
| $CaCl_2 \times 2 H_2O$ | 0.02 g |

whereby the ingredients are dissolved in 1 liter of deionized water which contained 10 ml of a trace element solution with the composition

| | |
|---|---|
| EDTA (Titriplex III) | 500 mg |
| $FeSO_4 \times 7\ H_2O$ | 200 mg |
| $ZnSO_4 \times 7\ H_2O$ | 10 mg |
| $MnCl_2 \times 4\ H_2O$ | 3 mg |
| $H_3BO_3$ | 30 mg |
| $CoCl_2 \times 6\ H_2O$ | 20 mg |
| $CuCl_2 \times 2\ H_2O$ | 1 mg |
| $NiCl_2 \times 6\ H_2O$ | 2 mg |
| $Na_2MoO_4 \times 2\ H_2O$ | 3 mg |

whereby the ingredients are dissolved in one liter of deionized water, supplemented with 0.2% (weight/volume) of neutralized octanoic acid, which had been adjusted to pH 7, was inoculated in an aerated stirred kettle with 500 ml of a stationary [translator: in house?] pre-culture of the strain Pseudomonas putida GPp104 (pHP1014:E156) in a complex medium consisting of "beef extract" (3 g) and peptone (5 g) dissolved in one liter of deionized water. After 12 and 24 hours of cultivation at 30°C, 0.5% (weight/volume) of neutralized levulinic acid were added in each case. Cell harvesting took place after a total of 48 hours of cultivation.

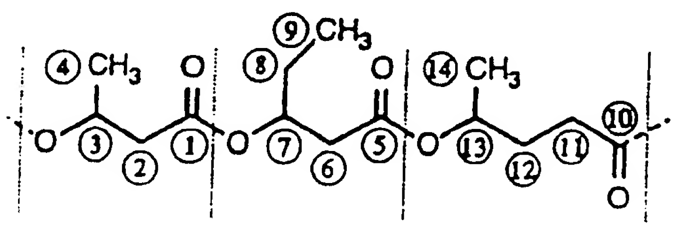
The analysis of the polymer, that was accumulated by the cells, resulted in a polyester concentration of 15% by weight of the dry mass of the cells. The polymer consisted of approximately 11 mol% of 3-hydroxybutyric acid, approximately 59 mol% of 3-hydroxyvaleric acid, approximately 15 mol% 4-hydroxyvaleric acid, approximately 10 mol% of 3-hydroxyhexanoic acid and approximately 5 mol% of 3-hydroxyoctanoic acid.

## Example 2

The procedure was followed as indicated in Example 1, except that use was made of the strain Alcaligenes eutrophus PHB$^{-}$4 (pHP1014::E156) instead of Pseudomonas putida GPp104 (pHP1014: :E156) and that, instead of neutralized octanoic acid, 0.3% neutralized gluconic acid was offered as the carbon source in addition to levulinic acid.

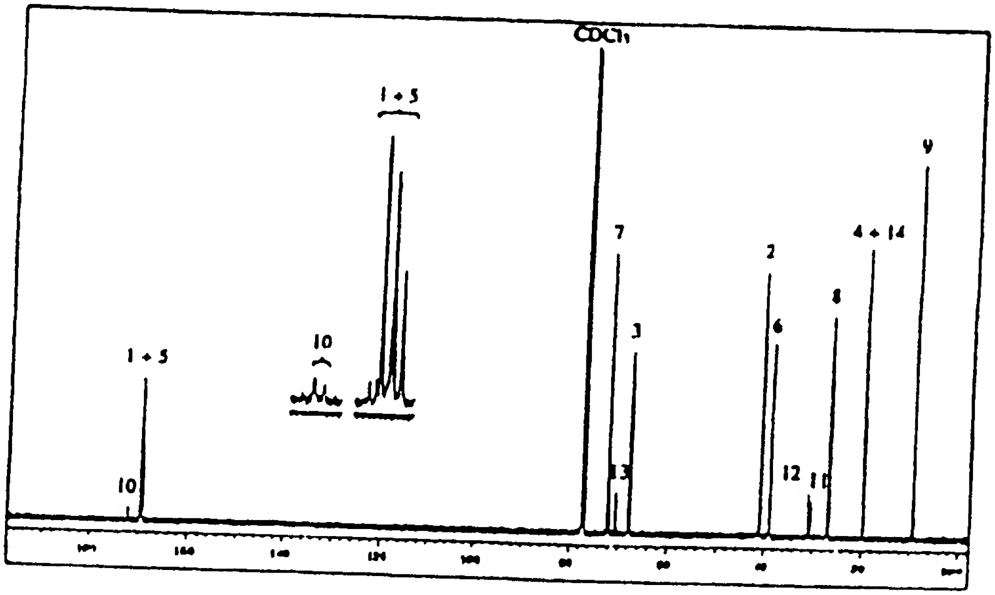
The analysis of the polymer, that was accumulated by the cells, resulted in a polyester concentration of 31% by weight of the dry mass of the cells. The polymer consisted of approximately 55 mol% of 3-hydroxybutyric acid, approximately 36 mol% of 3-hydroxyvaleric acid and approximately 9 mol% of 4-hydroxyvaleric acid.

The polyesters had, for example, the analytical data that are shown in the following section, whereby "A" reproduces the $^{13}$C-NMR spectrum and "B" reproduces the $^{1}$H-NMR spectrum. The signal assignment is found on the basis of the numbering which is indicated in the structural formula of poly(3HB-co-3HV-co-4HV) which is shown. The gas chromatogram after methanolysis of this polyester is then shown.

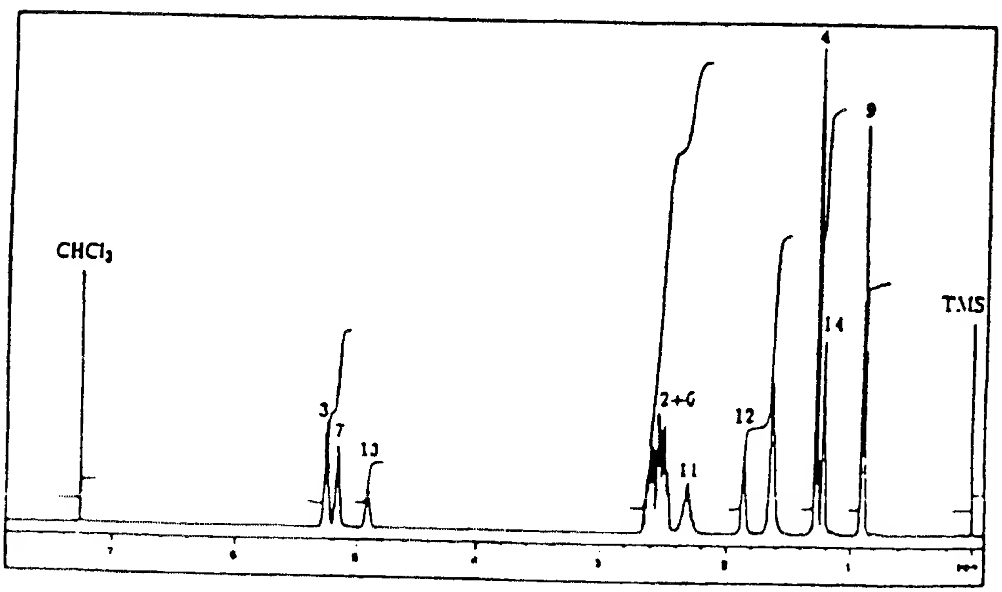


3HB 3HV 4HV

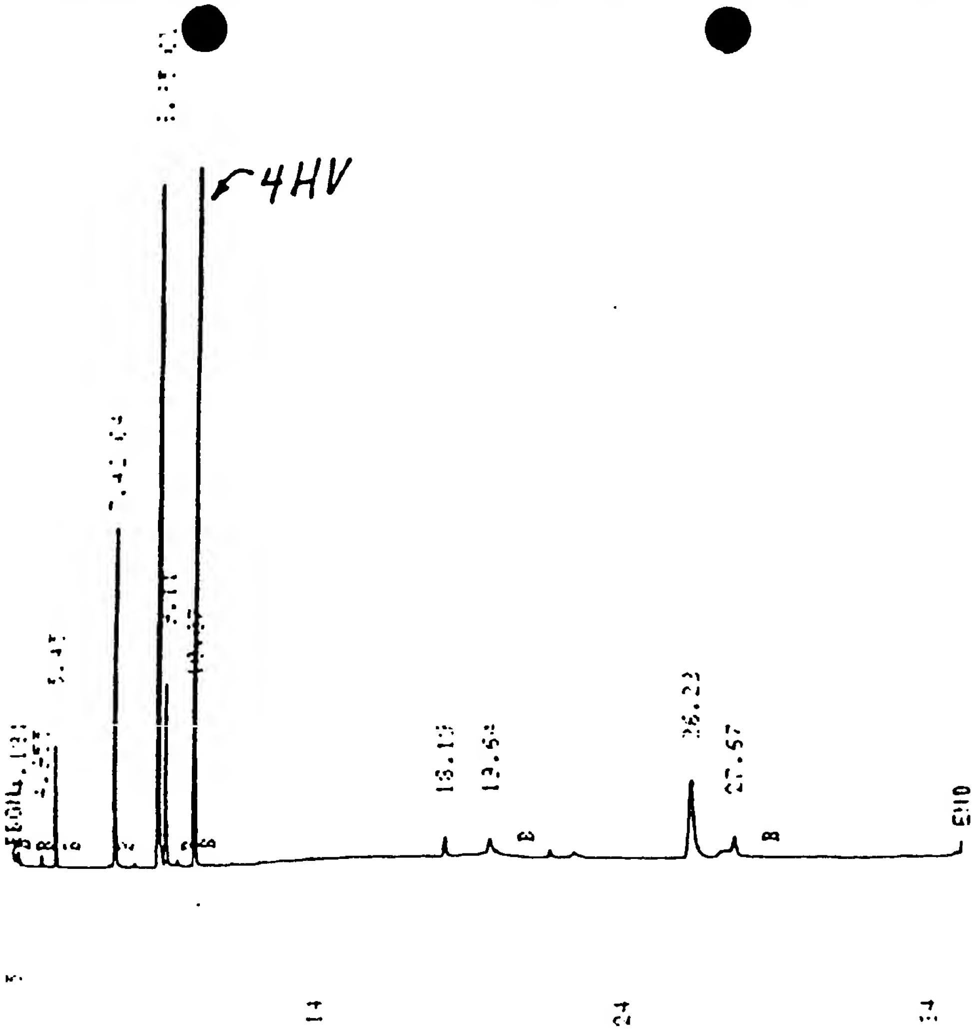
Structural formula of poly(3-hydroxybutyric acid-*co*-3-hydroxy-valeric acid-*co*-4-hydroxyvaleric acid) [poly(3HB-*co*-3HV-*co*-4HV)]




$^{13}$C-NMR-spectrum of poly(3HB-*co*-3HV-*co*-4HV)




$^{1}$H-NMR spectrum of the purified PHF from *A. eutrophus*




**Gas chromatogram of the purified PHF from A. eutrophus**

## Example 3

The procedure was followed as indicated in Example 2, except that a third portion of 0.5% (weight/volume) of levulinic acid was added after 36 hours and cultivation took place for a further 24 hours.

The analysis of the polymer, that was accumulated by the cells, resulted in a polyester content of 35% by weight of the dry mass of the cells. The polymer consisted of approximately 43 mol% of 3-hydroxybutyric acid, approximately 45 mol% of 3-hydroxyvaleric acid and approximately 12 mol% of 4-hydroxyvaleric acid.

## Example 4

The procedure was followed as indicated in Example 2, except that the volume of the inoculum from the pre-culture amounted to only 3 ml and that, as the main culture, inoculation took place with 50 ml of the aforementioned mineral salt medium in a 500 ml erlenmeyer flask. The flasks were then shaken aerobically for 72 hours before cell harvesting took place.

The analysis of the polymer, that was accumulated by the cells, resulted in a polyester content of approximately 25% by weight of the dry mass of the cells. The polymer consisted of approximately 61 mol% of 3-hydroxybutyric acid, approximately 32 mol% of 3-hydroxyvaleric acid and approximately 7 mol% of 4-hydroxyvaleric acid.

## Example 5

Cells of Alcaligenes eutrophus PHB$^-$4 (pHP1014::E156) from 50 ml of an aerobic pre-culture, that was 15 hours old, in the complex medium, that was designated in Example 1, were harvested by centrifugation, washed with sterile 0.9% sodium chloride solution and transferred to 50 ml of a modified mineral medium (as described in Example 1, but without $NH_4Cl$), which contained 1% (weight/volume) of levulinic acid and were shaken aerobically for 72 hours.

The analysis of the polymer, that was accumulated by the cells, resulted in a polyester content of approximately 37% by weight of the dry mass of the cells. The polymer consisted of approximately 37 mol% of 3-hydroxybutyric acid, approximately 50 mol% of 3-hydroxyvaleric acid and approximately 13 mol% of 4-hydroxyvaleric acid.

## Example 6

8 l of a mineral salt medium (Schlegel et al., 1961) with the composition

| | |
|---|---|
| $Na_2HPO_4 \times 12\ H_2O$ | 9.0 g |
| $KH_2PO_4$ | 1.5 g |
| $NH_4Cl$ | 0.5 g |
| $MgSO_4 \times 7\ H_2O$ | 0.2 g |
| $CaCl_2 \times 2\ H_2O$ | 0.02 g |
| Fe(III)$NH_4$ citrate | 0.0012 g |

which had been dissolved in one liter of deionized water which contained 10 ml of a trace element solution with the composition

| | |
|---|---|
| EDTA (Titriplex III) | 500 mg |
| $FeSO_4 \times 7\ H_2O$ | 200 mg |
| $ZnSO_4 \times 7\ H_2O$ | 10 mg |
| $MnCl_2 \times 4\ H_2O$ | 3 mg |
| $H_3BO_3$ | 30 mg |
| $CoCl_2 \times 6\ H_2O$ | 20 mg |
| $CuCl_2 \times 2\ H_2O$ | 1 mg |
| $NiCl_2 \times 6\ H_2O$ | 2 mg |
| $Na_2MoO_4 \times 2\ H_2O$ | 3 mg |

which had been dissolved in one liter of deionized water which was supplemented with 0.3% (weight/volume) of neutralized octanoic acid plus 0.1% (weight/volume) of neutralized precursor substrate and adjusted to pH 7, was inoculated with 500 ml of a stationary [translator: in-house?] pre-culture of the strain Pseudomonas putida GPp104 (pHP1014::E156) in a complex medium

12 and 36 hours in each case, 0.5% of precursor substrate was re-fed in and harvesting took place after a further 36 hours. The fermentation reactor was stirred at 500 rpm during cultivation and it was aerated at the rate of 800 ml of air per minute.

1. Incorporation of 5-hydroxyhexanoic acid

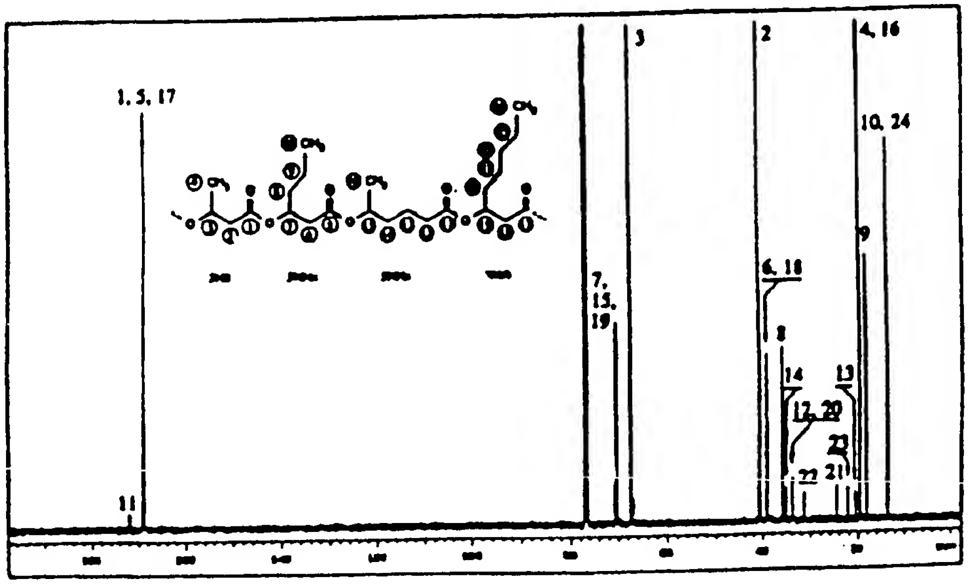
It was found that a recombinant strain of the PHF-free mutant GPp104 of Pseudomonas putida, which contains and expresses the gene of the PHF synthase from Thiocapsa pfennigii, can synthesize a copolyester that contains the new component 5-hydroxyhexanoic acid.

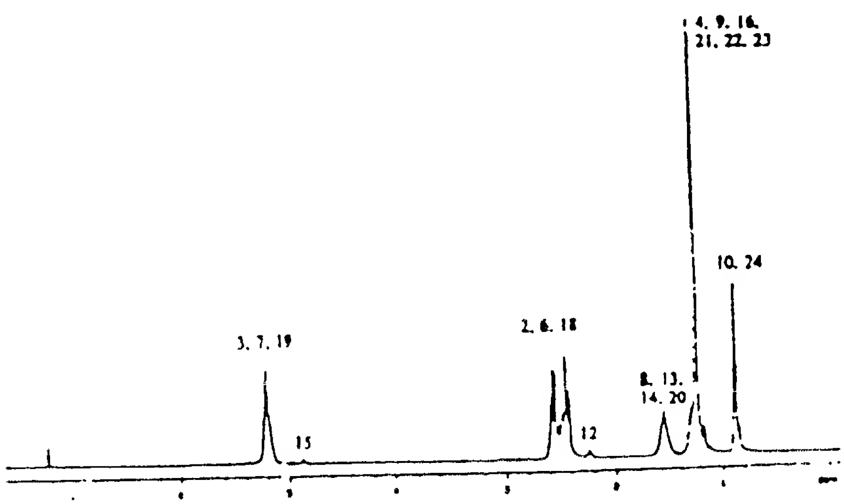
After methanolysis, the detection of 5HHx as a component took place gas chromatographically both in lyophilized cells and in the isolated and purified polyester. The isolated and purified polyester was subjected to $^{13}$C-NMR and $^{1}$H-NMR analysis and the incorporation of 5HHx was confirmed as a result of this.

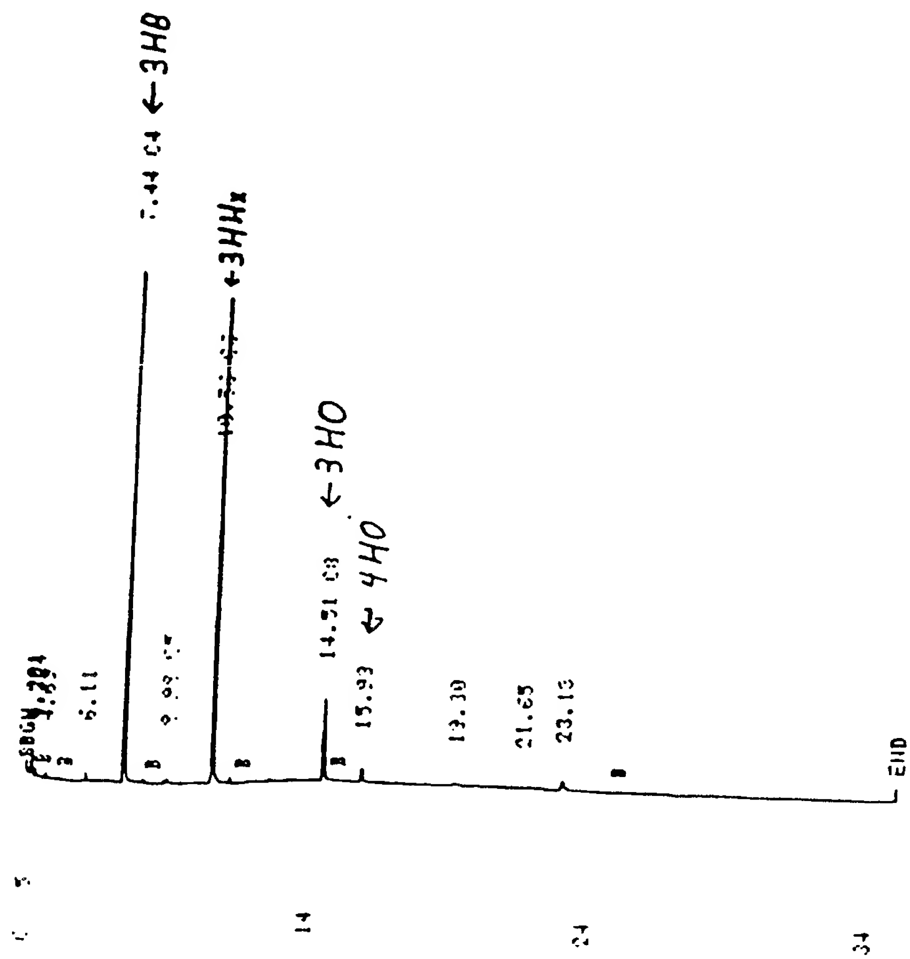
The preparation of 5-hydroxyhexanoic acid took place starting from 4-acetylacetic acid which had been quantitatively reduced using $NaBH_4$.

The analysis of the polymer, that was accumulated by the cells, resulted in a polyester content of approximately 36% by weight of the dry mass of the cells. The polymer consisted of approximately 71 mol% of 3-hydroxybutyric acid, approximately 4 mol% of 3-hydroxyhexanoic acid and approximately 23 mol% of 5-hydroxyhexanoic and approximately 2 mol% of 3-hydroxyoctanoic acid (and, in addition, a minimal quantity of 4-hydroxyoctanoic acid).

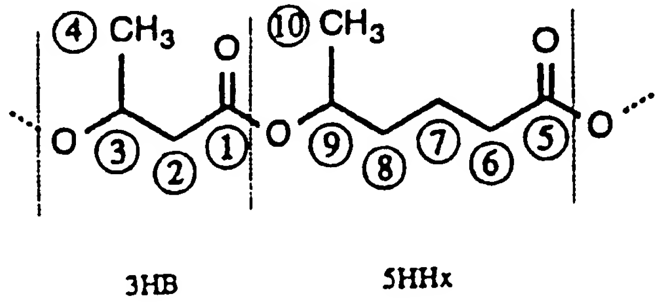
The polyesters exhibit, for example, the analytical data that are shown in the following section, whereby "A" reproduces the $^{13}$C-NMR spectrum and "B" reproduces the $^{1}$H-NMR spectrum. The signal assignment is found on the basis of the numbering which is indicated in the structural formula of poly(3HB-co-3HHx-co-5HHx-3HO) which is shown. The GC analysis after


1, 5, 17
11
7,
15,
19
3
2
6, 18
8
14
12, 20
22
23
21
13
4, 16
10, 24
9


4, 9, 16,
21, 22, 23
10, 24
3, 7, 19
2, 6, 18
8, 13,
14, 20
15
12

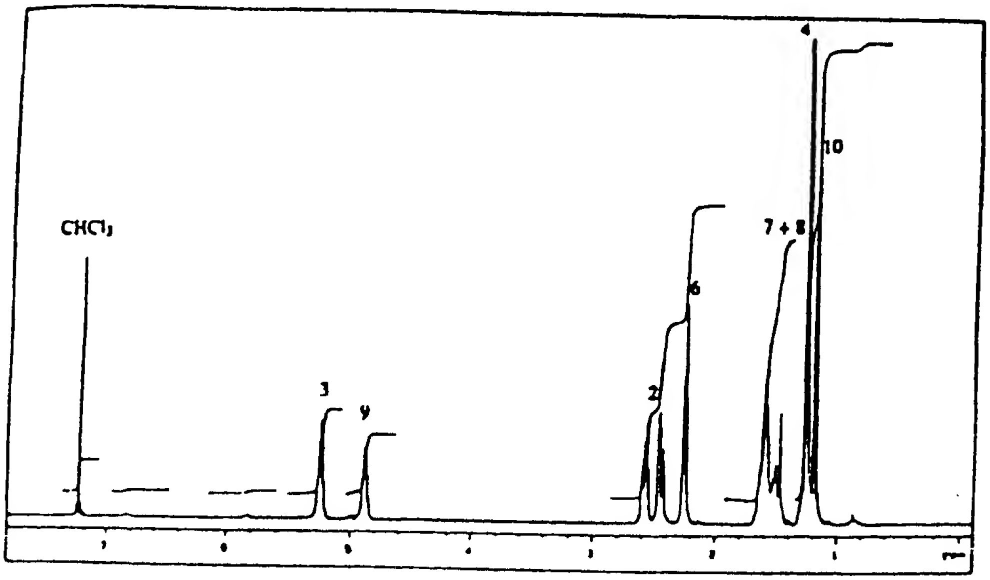


However, it is also possible to obtain a poly(3HB-co-5HHx) PHF whose spectroscopic and GC data are shown in the following section. The polyesters exhibit, for example, the analytical data that are shown in the following section, whereby "A" reproduces the $^{13}$C-NMR spectrum and "B" reproduces the $^{1}$H-NMR spectrum. The signal assignment is found on the basis of the numbering

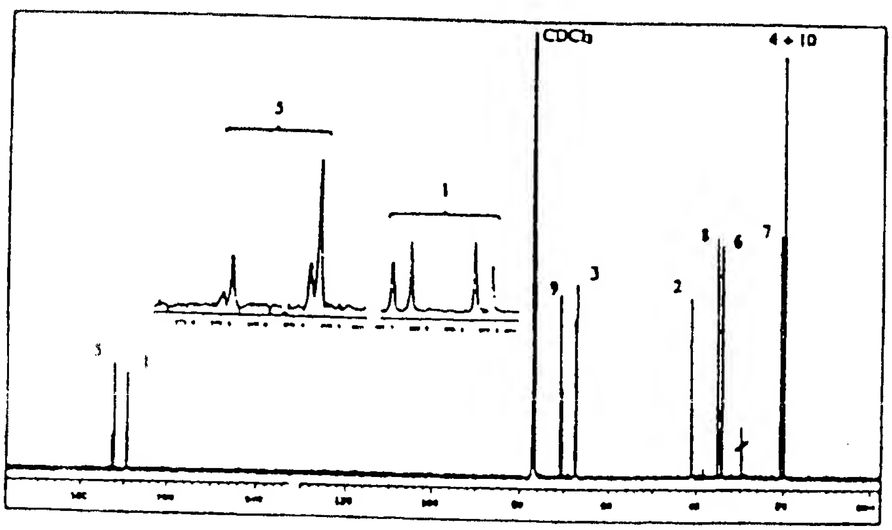


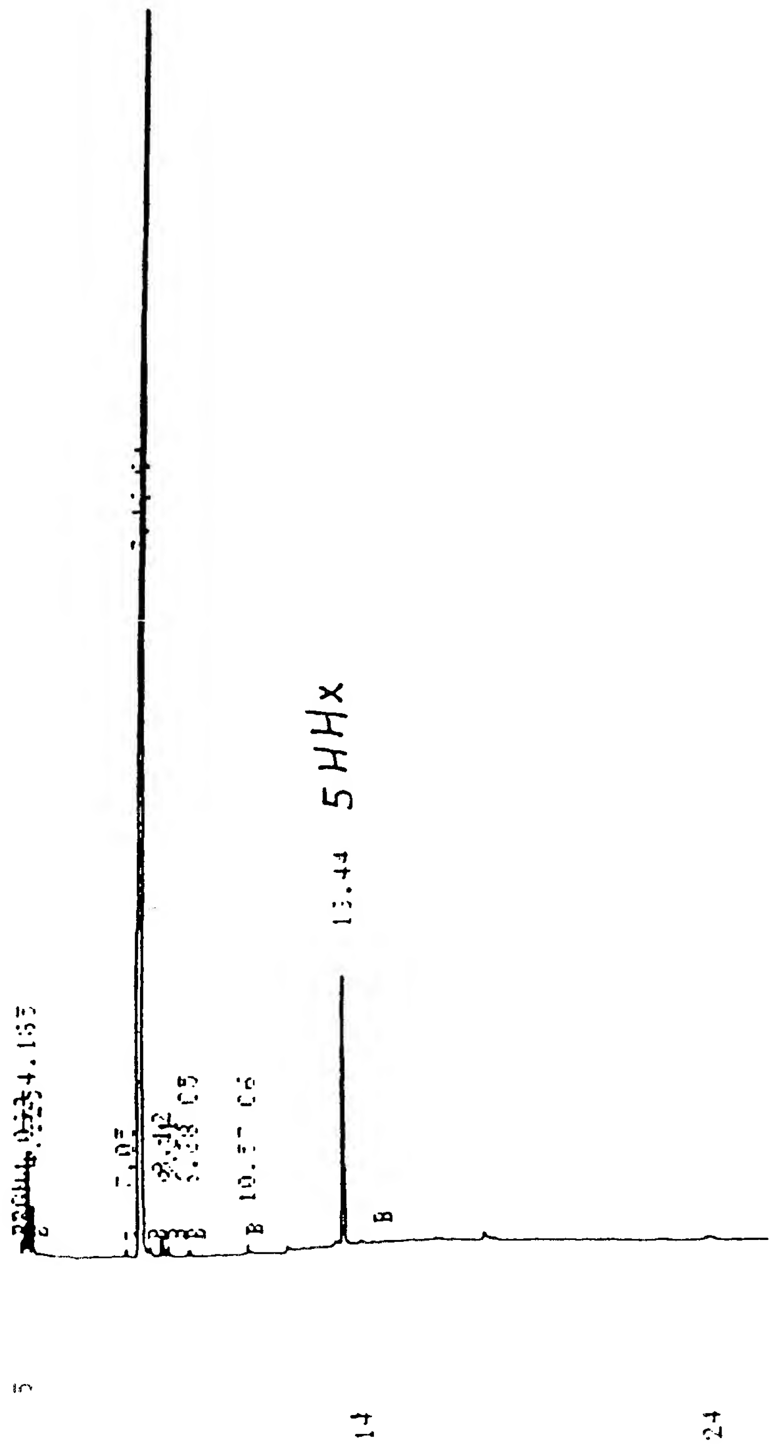

Structural formula of poly(3-hydroxybutyric acid-*co*-5-hydroxy-hexanoic acid) [poly(3HB-*co*-5HHx)].

A




B






Gas chromatogram of poly(3HB-co5HHx)

## 2. Incorporation of 4-hydroxyheptanoic acid

It was found that a recombinant strain of the PHF-free mutant GPp104 of Pseudomonas putida, which contained and expressed the gene of the PHF synthase from Thiocapsa pfennigii, can synthesize a copolyester that contains the new component 4-hydroxyheptanoic acid (4HHp).

After methanolysis, the detection of 4HHp took place gas chromatographically both in lyophilized cells and also in the isolated and purified polyester.

The preparation of 4-hydroxyheptanoic acid took place via hydrolysis of g-heptolactone with NaOH.

The analysis of the polymer, that was accumulated by the cells, resulted in a polyester content of 39% by weight of the dry mass of the cells. The polymer consisted of 43 mol% of 3-hydroxybutyric acid, 16 mol% of 3-hydroxyvaleric acid, 27 mol% of 3-hydroxyhexanoic acid, 5 mol% of 3-hydroxyheptanoic acid, 6 mol% of 4-hydroxyheptanoic acid and 3-hydroxyoctanoic acid.

## 3. Incorporation of 4-hydroxyoctanoic acid

It was found that a recombinant strain of the PHF-free mutant GPp104 of Pseudomonas putida, which contained and expressed the gene of the PHF synthase from Thiocapsa pfennigii, can synthesize a copolyester that contains the new component 4-hydroxyoctanoic acid (4HHp).

After methanolysis, the detection of 4HO gas took place chromatographically both in lyophilized cells and also in the isolated and purified polyester. Because of the small proportion of 4HO, NMR spectroscopic investigations were not possible.

The preparation of 4-hydroxyoctanoic acid took place via hydrolysis of g-octalactone with NaOH

The analysis of the polymer, that was accumulated by the cells, resulted in a polyester content of approximately 18% by weight of the dry mass of the cells. The polymer consisted of approximately 75 mol% of approximately [sic] 3-hydroxybutyric acid, approximately 22 mol% of 3-hydroxyhexanoic acid, approximately 1.5 mol% of 3-hydroxyoctanoic acid and approximately 3 mol% of 4-hydroxyoctanoic acid.

## Example 7

In order to obtain the new polyesters on a smaller scale, e.g. for analytical and test purposes or in order to test strains in terms of their capacity for being able to bio-synthesize these new polyesters, one generally proceeded as follows:

Cells of Pseudomonas putida GPp104 (pHP1014::E156) from 50 ml of an aerobic pre-culture, that was 15 hours old, in the complex medium, that was designated in Example 1, were harvested by centrifugation, washed with sterile 0.9% sodium chloride solution and transferred to 50 ml of a modified mineral medium (as described in Example 1, but without $NH_4Cl$) and shaken aerobically for 72 hours.

Using 5-hydroxyhexanoic acid, which was added in portions (0.25 plus 0.25 plus 0.5%) to, in total, a concentration of 1%, P. putida GPp104 (pHP1014::E156) accumulated PHF up to a proportion of maximally 40% of the dry mass of the cells and comprised approximately [sic] 3-hydroxybutyric acid (approximately 50 to 80 mol%), 3-hydroxyhexanoic acid (approximately 3 to 10 mol%) and 5-hydroxyhexanoic acid (approximately 10 to 30 mol%).

Using 4-hydroxyheptanoic acid, which was added in portions (0.25 plus 0.25 plus 0.5%) to, in total, a concentration of 1%, P. putida GPp104 (pHP1014::E156) accumulated PHF up to a proportion of maximally 40% of the dry mass of the cells and comprised approximately [sic] 3-hydroxybutyric

hydroxyheptanoic acid (approximately 1 to 5 mol%) and 4-hydroxy-heptanoic acid (approximately 3 to 10 mol%).

Using 4-hydroxyoctanate, which was fed in four times with a concentration of 0.2%, P. putida GPp104 (pHP1014::E156) accumulated PHF up to a proportion of maximally approximately 50% of the dry mass of the cells and comprised 3-hydroxybutyric acid (approximately 70 to 90 mol%), 3-hydroxyvaleric acid (approximately 1 to 5 mol%), 3-hydroxyhexanoic acid (approximately 10 to 20 mol%), 3-hydroxyoctanoic acid (approximately 1 to 5 mol%) and 4-hydroxyoctanoic acid (approximately 0.5 to 4 mol%).

## Example 8

In order to obtain the new polyesters on a smaller scale for analytical and/or test purposes or in order to test the strains in terms of their capacity for being able to bio-synthesize these new polyesters, one also proceeded alternatively as follows.

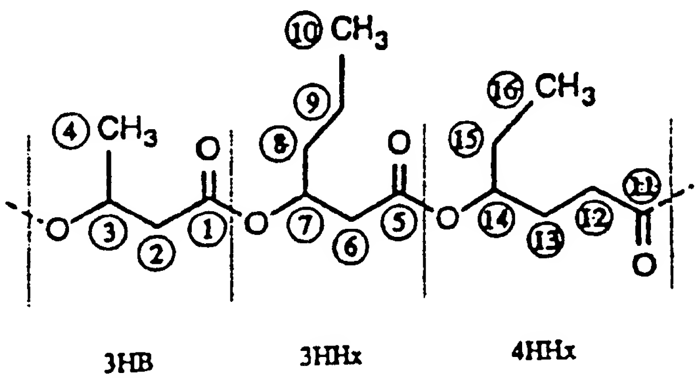
One proceeded as indicated in Example 1, except that the volume of the inoculum from the pre-culture amounted to only 3 ml and that, as the main culture, inoculation was carried out with 50 ml of the aforementioned mineral salt medium in a 500 ml erlenmeyer flask. The flasks were then shaken aerobically for 72 hours before cell harvesting took place.

The results of the accumulation process (proportion of PHF on the dry mass of the cells and the composition of the polymer) were [recorded?] and varied within the framework which is described in Example 7.

## Example 9

Analogously to the Examples 1 through 7, the new bacterial strains Pseudomonas putida GPp104 (pHP1014::B28+), DSM No. 9417, and Alcaligenes eutrophus PHB$^{-}$4 (pHP1014::B28+), DSM No. 9418, were used as the production organisms.

In the case of the example, a copolyester of formula poly(3HB-co-3HHx-co-4HHx) could be prepared whose analytical data are reproduced in the following section. The polyesters exhibited, for example, the analytical data that are shown in the following section, whereby "A" reproduces the $^{13}$C-NMR spectrum and "B" reproduces the $^{1}$H-NMR spectrum. The signal assignment is found on the basis of the numbering which is indicated in the structural formula of poly(3HB-co-3HHx-co-4HHx) that is shown. The gas chromatogram after methanolysis is then shown.



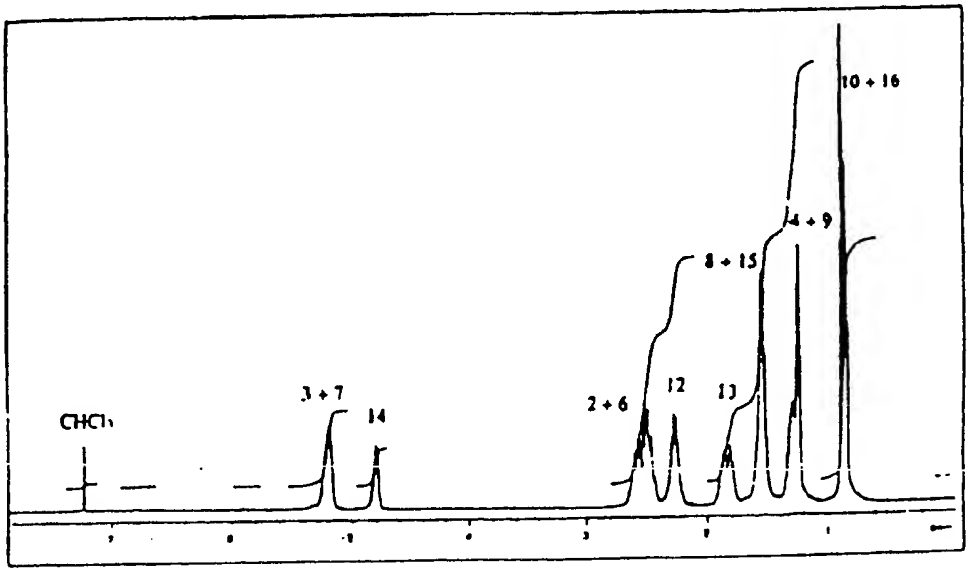

Structural formula of poly(3-hydroxybutyric acid-*co*-3-hydroxy-hexanoic acid-*co*-4-hydroxyhexanoic acid) [poly(3HB-*co*-3HHx-*co*-4HHx)].

**Chemical shifts of the $^{13}$C-NMR signals from poly(3HB-*co*-3HHx-*co*-4HHx).**

| | | Chemical shift | | |
|---|---|---|---|---|
| Monomer | Carbon | | [ppm] | |
| 3HB | 1 | 169.05 | - | 169.89 |
| | 2 | 40.84 | - | 40.91 |
| | 3 | 67.32 | - | 67.78 |
| | 4 | 19.70 | - | 19.83 |
| 3HHx | 5 | 169.05 | - | 169.89 |
| | 6 | 39.18 | - | 39.41 |
| | 7 | 70.34 | - | 70.77 |
| | 8 | 35.96 | - | 36.12 |
| | 9 | 18.30 | - | 18.34 |
| | 10 | | | 13.73 |
| 4HHx | 11 | 171.91 | - | 172.61 |
| | 12 | 30.46 | - | 30.50 |
| | 13 | 28.61 | - | 28.86 |
| | 14 | 74.66 | - | 74.95 |
| | 15 | 26.80 | - | 26.95 |
| | 16 | 9.38 | - | 9.42 |

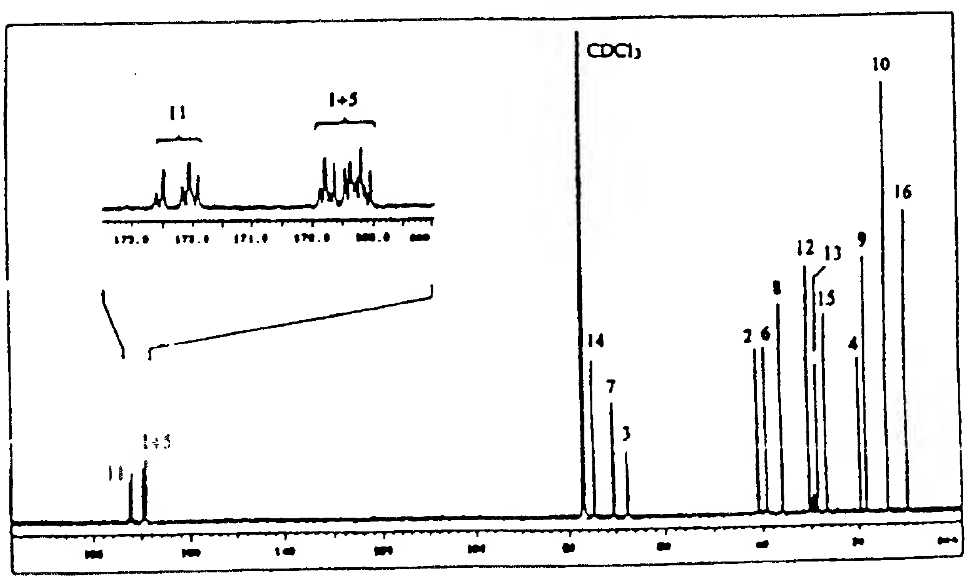
3HB: 3-hydroxybutyric acid; 3HHx: 3-hydroxyhexanoic acid; 4HHx: 4-hydroxyhexanoic acid.

A

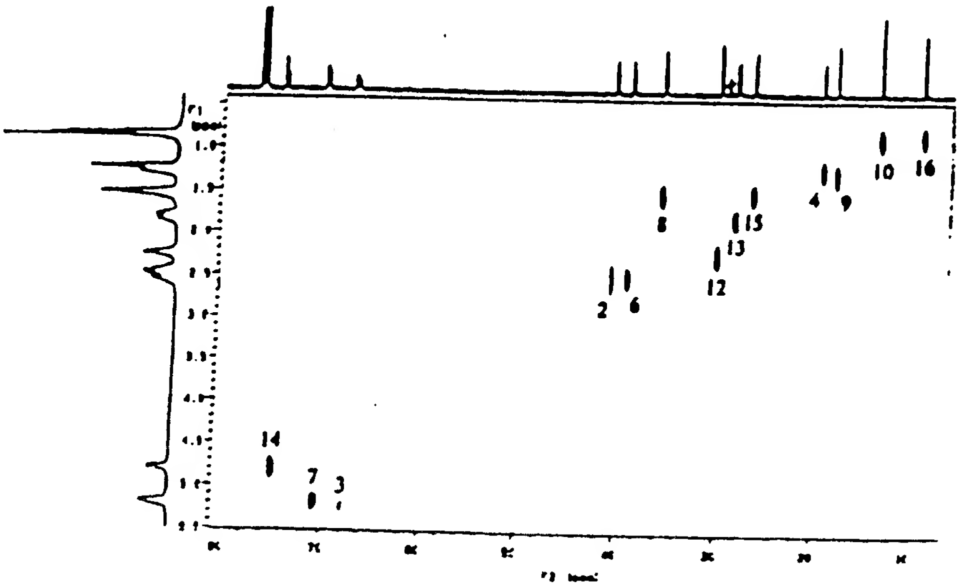


CHCl₃
3 + 7
14
2 + 6
12
13
8 + 15
4 + 9
10 + 16

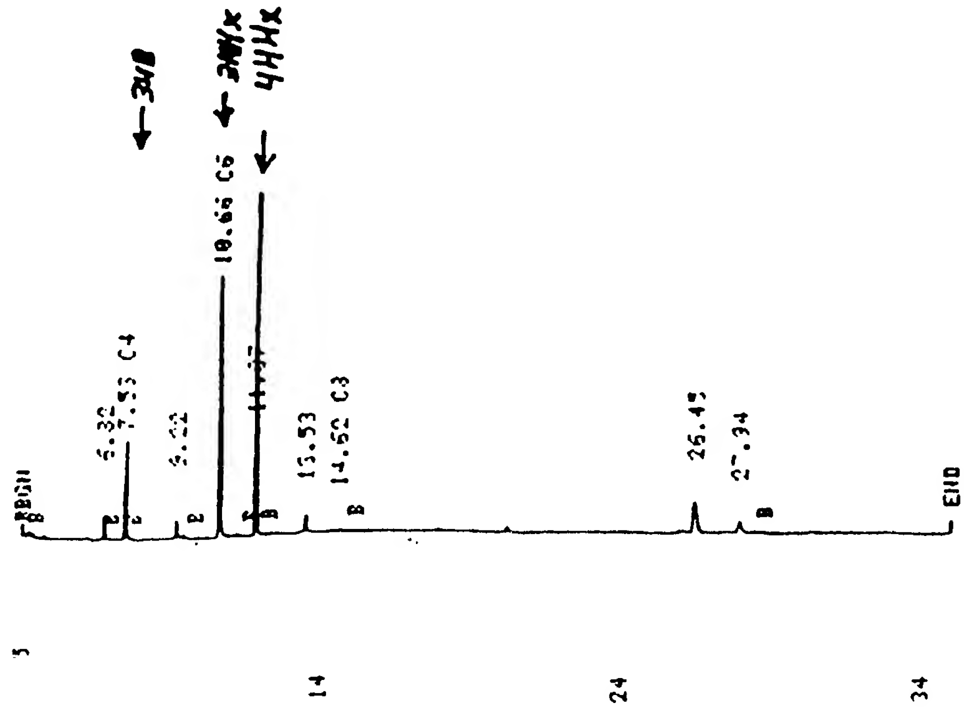

B



CDCl₃
11
1+5
14
7
3
2
6
8
12
13
15
4
9
10
16




NMR spectroscopic analysis of poly(3HB-*co*-3HHx-*co*-4HHx).




Gas chromatogram of poly(3HB-co-3HHx-co-4HHx)